



Increased egg-laying in *Orius insidiosus* (Hemiptera: Anthocoridae) fed artificial diet supplemented with an embryonic cell line

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Abstract

The insidious flower bug, *Orius insidiosus* (Say) (Hemiptera: Anthocoridae), can be reared on an artificial diet absent of insect components, but yolk production is reduced and resultant rates of oviposition and egg production are poor. Supplementing the artificial diet with cells from an embryonic cell line, IPLB-(PiE) of *Plodia interpunctella* (Hübner), enhanced oviposition rates. Ovipositional rates were evaluated using a short 7-day bioassay. Cells were separated from Grace's medium containing 10% fetal bovine serum, washed several times in Grace's medium alone, and then homogenized before they were incorporated into the diet. In addition to the cell-supplemented diet, several control diets were also tested: diet supplemented with Grace's medium alone, Grace's medium with fetal bovine serum, and conditioned Grace's medium (i.e., the medium the cells were cultured in). Adult predators were placed on the diets the third day after eclosion and allowed to feed for 6 days and provided with an oviposition substrate for 24 h on day 7. Egg production was significantly increased only in the cell-supplemented diet, a similar result that occurred when cumulative egg production was recorded during the life span of the females. Oviposition increased incrementally with the increase in concentration of cells added to the diet and was significantly better than control diet at 0.25 ml homogenized cells/1.2 ml diet (1.123×10^5 cells, 31 μ g protein) to 1.0 ml cells/1.2 ml diet (1.86×10^6 cells, 124 μ g protein). Although the cell line enhanced the rate of oviposition at a relatively low concentration of protein, we do not know if the protein or some other nutritional components of the cells were responsible for the increased oviposition rate. The cell line may be used to directly supplement the described diet for *O. insidiosus* and/or serve as a source of material that could simplify purification and identification of the cell component(s) responsible for stimulating oviposition. Additionally, the 7-day bioassay may be used to expedite identification of active fractions during chemical analysis of the cell line.

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1. Introduction

The insidious flower bug, *Orius insidiosus* (Say) (Hemiptera: Anthocoridae), is a common predator in sweet corn and field crops in eastern and central North America and is used in commercial flower and vegetable greenhouses for biological control of thrips, spider mites, aphids, insect eggs, small caterpillars, and other small insects (Association of Natural Bio-control Producers, 2001, <http://www.anbp.org>; Barber, 1936). Currently, eggs of the Mediterranean flower moth,

Ephestia kuehniella Zeller, are used as a food to rear *Orius* spp., but they are expensive. One very promising application would substitute eggs of *E. kuehniella* (Hübner) entirely with an artificial diet for rearing *Orius* spp. Although *O. insidiosus* can be reared on an artificial diet that is economical to produce (Weiru and Ren, 1989), the fecundity of the females is significantly less than those reared on Indian meal moth, *Plodia interpunctella* (Hübner) or *E. kuehniella* eggs (Ferkovich and Shapiro, 2003). Adding protein from eggs of *P. interpunctella* improved the rate of oviposition at levels that were significantly lower than those of commercial non-insect proteins. The approach of adding crude insect-derived materials to diet to improve growth, development, and fecundity of entomophagous insects appears

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to be generally successful (Grenier et al., 1994; Nettles, 1990).

Although many artificial diets developed for entomophagous insects can be amended with insect materials (e.g., hemolymph), the labor and problems (e.g., melinization) associated with incorporating insect tissues into diets is not practical (Grenier et al., 1994; Mitsuhashi and Oshiki, 1993; Nakahara et al., 1997; Nettles, 1990). A possible solution to this problem is supplementation of diets with insect cells produced in vitro. This approach has been tested on several parasitoids with promising results. Rotundo et al. (1988) reared a small number of the aphid endoparasitoid *Lysiphlebus fabarum* (Marshall) from first instar larva to adult stage in vitro, using a cell line derived from the dipteran *Ceratitis capitata* Wiedeman. Ferkovich et al. (1994) showed that several insect cell lines stimulated in vitro egg development of *Microplitis croceipes* (Cresson), an endoparasitoid of *Heliothis* species. Hu et al. (1999) reported that insect hemolymph could be replaced successfully with insect cell line-conditioned medium incorporated into artificial diets to support the growth and development of the egg parasitoid *Edovum puttleri* (Grissell). Conceivably, cell lines could also be used to supply nutritional factors for growth and reproduction of predators such as *O. insidiosus*.

In this study, we investigated the potential of enhancing oviposition in *O. insidiosus* fed an artificial diet supplemented with an embryonic cell line (PiE).

2. Materials and methods

2.1. Diets

2.1.1. *Plodia* eggs

Eggs from *P. interpunctella* were used as a dietary standard against which to gauge oviposition of insects within each assay. Eggs were obtained from a laboratory colony of *P. interpunctella* reared as described by Silhacek and Miller (1972). Eggs were collected within 1 h after oviposition, held at 4 °C for 24 h, and then stored at –80 °C until used in our experiments.

2.1.2. Artificial diet

Artificial diet was prepared under aseptic conditions in a clean room and encapsulated in stretched Parafilm using a diet encapsulation apparatus (Analytical Research Systems, Gainesville, FL) described by Ferkovich et al. (1999). Artificial diet was prepared as described for rearing *Orius sauteri* (Weiru and Ren, 1989), and consisted of 0.33 g brewer's yeast, 0.03 g sucrose, 0.18 g soy protein acid hydrolysate, 3.8 mg of 99% palmitic acid (all from Sigma, St. Louis, MO), 0.04 g chicken egg yolk, and 0.08 g honey in 1.2 ml of distilled water. Palmitic acid was mixed with the egg yolk component before adding it to the diet.

2.1.3. PiE cells

The PiE cell line-supplemented diet was prepared using an embryonic cell line (IPLB-PiE) originally derived from eggs of *P. interpunctella*, using methods previously described (Lynn, 1996). The cells were cultured in TNM-FH insect medium (Sigma, St. Louis, MO), which is modified Grace's medium supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO). For larger-scale culture of the cell lines, cells were grown in 250 ml magnetic spinner flasks (Bellco Glass, Vineland, NJ) at 24.9 °C. The cell line suspension was centrifuged at 1370g in a graduated conical centrifuge tube to obtain a soft pellet of cells and the supernatant was saved to prepare the *GMCond* diet in the first bioassay. PiE cell line-supplemented diet which was tested in the first and second bioassay was prepared as follows: aliquots of the cellular pellet were transferred to graduated conical tubes, diluted to 1.2 ml with distilled water, and homogenized with a hand-held homogenizer. Diet ingredients were added to each of the tubes and sonicated for 10 s using a Polytron unit (Model W-375, Heat Systems-Ultrasonic, Plain View, NY) to thoroughly mix the cellular material in the diet. Protein in the supernatant and cell homogenate was analyzed using the Lowry procedure for soluble proteins (Protein Assay Kit, Sigma, St. Louis, MO).

2.2. Bioassay

Newly emerged adults of a Florida strain of *O. insidiosus* (<24 h after eclosion) were obtained from a commercial producer of beneficial insects (Entomos, Gainesville, FL). The insects were placed on the diets on the third day after eclosion. Each replicate consisted of six females and four males in a 100 ml plant tissue culture jar (Sigma, St. Louis, MO) with four jars per treatment. Each jar contained one Parafilm capsule of water (50 µl), two capsules of treatment diet (each 50 µl), one 7-cm section of green bean pod for oviposition, and three crumpled strips of wax paper (5 mm × 80 mm) as substrates. *Plodia* eggs and domes of water and diet were replaced daily and mortality was recorded. At the end of the sixth day, one 7-cm section of green bean pod, used as a substrate for oviposition, was placed in each jar for 24 h. Eggs deposited in the green beans were then counted under a microscope. The insects were held in a growth chamber at 25.5 ± 1 °C, with 75 ± 5% RH, and a photoperiod of 15:9 (L:D) h.

Diet treatments consisted of the following: *Plodia* Egg—75 *P. interpunctella* eggs (3 mg). *Artificial Diet*—control diet with no additional substances. *Experimental diets*—artificial diet with a supplemental medium or cells, incorporated as described. Although eggs from *E. kuehniella* are generally used by commercial producers, we used eggs from another pyralid moth, *P. interpunctella*, because *O. insidiosus* also feeds readily on their

eggs and *P. interpunctella* are reared in our laboratory under controlled conditions.

2.2.1. First test

The purpose of the first bioassay was to determine whether incorporating Grace's medium and cell line-conditioned medium (i.e., the medium the cells were grown in) into diet had any potential oviposition-enhancing effects on females in comparison with cells incorporated into diet. The experimental diets were: (1) *Plodia Eggs*; (2) *Artificial Diet*; (3) *GM*—*Artificial Diet* prepared with unconditioned Grace's modified culture medium; (4) *GMFBS*—*Artificial Diet* with 10% FBS added; (5) *GMCond*—*Artificial Diet* prepared with Grace's modified culture medium in which *PiE* cells had been cultured; and (6) *PiE Cells*—*Artificial Diet* prepared with 0.3 ml of homogenized cells (20.3 µg protein/1.2 ml diet). In an ancillary test, predators were maintained on the same treatment diets until the females in all of the treatments ceased egg laying (22 days), and the cumulative number of eggs oviposited was determined. Green beans were removed every other day to determine eggs oviposited and replaced with fresh beans. *Plodia* eggs and domes of water and diet were replaced on the same days that the green beans were removed.

2.2.2. Second test

The objective of the second test was to find out if the cell line would affect oviposition rate in a dose–response manner. The treatment diets were (1) *Plodia Eggs*; (2) *Artificial Diet*; and (3) *PiE Cells*—artificial diet prepared with 0.25, 0.5, 0.75, and 1.0 ml of homogenized cells, resulting in 31, 62, 93, and 124 µg/1.2 ml diet, respectively.

2.3. Data analysis

Each treatment was replicated four times with six ♀ and four ♂/replicate. The egg counts were adjusted for female mortality within each treatment and the means and standard errors were calculated from the four replications. Data were analyzed by ANOVA techniques using StatMost software (Dataxiom Software). Percent survival of females in each treatment was transformed using square root transformation and arc sine transformation. Newman–Keuls multiple-range test was used to compare all of the diet treatment means, including the control diet, with every other mean.

3. Results

3.1. First test

In the 7-day bioassay, only the *PiE Cells* diet significantly increased the oviposition rate relative to the

Artificial Diet, and the Grace's control diets (*GM*, *GMFBS*, and *GMCond*) ($F = 4.31$; $df = 4$; $P = 0.016$) (Fig. 1). In the accessory test, which measured cumulative egg production in each treatment during the lifetime of the females, oviposition (mean cumulative eggs/♀) on each diet over 22 days was as follows: *Plodia Eggs*, 43.4 ± 4.6 ; *Artificial Diet*, 15.6 ± 1.7 ; *GM*, 14.4 ± 0.6 ; *GMFBS*, 16.5 ± 1.1 ; *GMCond*, 17.5 ± 1.6 ; and *PiE Cells*, 24.12 ± 1.5 . Females fed the *PiE Cells* diet oviposited significantly more eggs than those fed the *Artificial Diet* ($P < 0.05$), and the Grace's control diets, *GM* ($P < 0.01$), *GMFBS* ($P < 0.05$), and *GMCond* ($P < 0.05$) ($F = 4.59$; $df = 4$; $P = 0.013$). The survival of females on the *PiE Cells* diet was significantly better than the *Artificial Diet* and all of the Grace's control diets, *GM*, *GMFBS*, and *GMCond* ($F = 6.84$; $df = 4$; $P = 0.013$) and was 85% of the *Plodia Eggs* (Table 1).

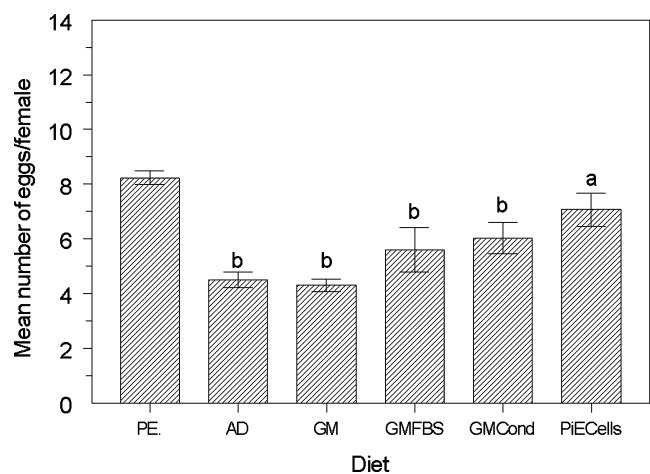


Fig. 1. Comparison of mean number of eggs oviposited per 24 h after females fed on *Plodia Eggs* (PE), *Artificial Diet* (AD), *GM*, *GMFBS*, *GMCond*, and *PiE Cells* for 6 days. Bars with the same letter are not significantly different (Newman–Keuls method, $P > 0.05$); error bars = standard error.

Table 1

Survival of female predators fed on artificial diet, Grace's control diets, and *PiE Cells* shown in Fig. 1

Treatment	% Survival \pm SE ^{a,b}	% of <i>Plodia Eggs</i> ^c
<i>Plodia eggs</i>		
<i>Artificial Diet</i>	37.5 \pm 4.2	47 b
<i>GM</i>	41.6 \pm 4.9	53 b
<i>GMFBS</i>	50.0 \pm 0.0	63 b
<i>GMCond</i>	50.1 \pm 6.9	63 b
<i>PiE Cells</i> (1.0 ml/ml diet)	67.0 \pm 0.0	85 a

^a Means \pm SE of {# females remaining alive on treatment/# females remaining alive on *Plodia Eggs* \times 100}; $n = 4$.

^b Means in a column followed by the same letter are not significantly different (Newman–Keuls method, $P > 0.05$). Error bars = standard error. Percent data for survival of females in each treatment was transformed using square root transformation and arc sine transformation.

^c Percent survival on *Plodia Eggs* averaged 79.0 ± 4.0 SE in Fig. 1.

3.2. Second test

Females fed on the *PiE Cells* diet for 7 days laid significantly more eggs/♀ at the 0.25, 0.5, 0.75, and 1.0 ml doses of cells per 1.2 ml of diet than those that fed on the *Artificial Diet* ($F = 17.97$; $df = 4$; $P = 0.0001$) (Fig. 2). Mean eggs produced/♀ was highest at the 0.5 and 0.75 ml levels of cells then declined at the 1.0 ml level. The survival of females on the *PiE Cells* diet at the 1.0 ml level was significantly better than the *Artificial Diet* and the lower doses of cells ($F = 13.02$; $df = 4$; $P = 0.0001$) and was 96% of the *Plodia Eggs* (Table 2).

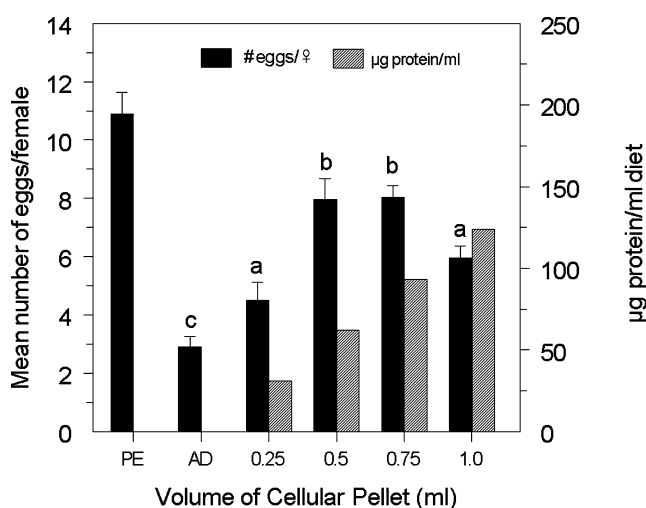


Fig. 2. Comparison of mean number of eggs oviposited per 24 h after females fed for 6 days on *Plodia Eggs* (PE), *Artificial Diet* (AD), and *PiE Cells* with increasing dose of cellular pellet per ml of diet. Quantity of protein in each volume is given on right axis. Bars with the same letter are not significantly different (Newman–Keuls method, $P > 0.05$). Error bars = standard error.

Table 2

Survival of female predators fed on artificial diet and supplemented with *PiE* cells shown in Fig. 2

Treatment	% Survival \pm SE ^{a,b}	% of <i>Plodia Eggs</i> ^c
<i>PiE Cells</i> (ml/ml diet)		
0 (<i>Artificial Diet</i>)	54.2 \pm 4.2	68 b
0.25	50.0 \pm 0.0	63 b
0.5	41.7 \pm 4.8	53 b
0.75	54.2 \pm 4.2	68 b
1.0	79.2 \pm 4.1	96 a

^a Means \pm SE of {# females remaining alive on treatment/# females remaining alive on *Plodia Eggs* \times 100}; $n = 4$.

^b Means in a column followed by the same letter are not significantly different (Newman–Keuls method, $P > 0.05$). Error bars = standard error. Percent data for survival of females in each treatment was transformed using square root transformation and arc sine transformation.

^c Percent survival on *Plodia Eggs* averaged and 79.2 ± 4.1 SE in Fig. 2.

4. Discussion

Cells of the embryonic *PiE* cell line made a significant improvement in egg production when added to the artificial diet. To date, the use of insect cell lines for in vitro culture of insects has been reported in several species of parasitoids, but this is the first study on the use of a cell line to supplement the diet of a predator. The studies on parasitoids focused on improving the growth and development of two endoparasitoids, *L. fabarum* (Marshall) (Rotundo et al., 1988) and *M. croceipes* (Ferkovich and Oberlander, 1991; Ferkovich et al., 1994) a pupal ectoparasitoid, *Diapetiorpha introita* (Cresson) (Ferkovich et al., 1999), and an egg parasitoid, *E. puttleri* (Hu et al., 1999). Hu et al. (1999) tested cell lines from 11 insect species on *E. puttleri* and found that development from the egg to pupal stage was best with artificial diet prepared with cell line-conditioned media (i.e., the medium the cells were grown in, but with the cells removed) from an embryonic cell line derived from the parasitoid's host. In this study, we used an embryonic cell line derived from eggs of *P. interpunctella*. Females have naturally high fecundity on *P. interpunctella* eggs (unpublished observations), but we saw no contribution to the rate of egg laying by the pre-conditioned medium or any of the other cell culture additives; only the homogenized whole cells had an effect. Apparently, the oviposition-promoting material is not released into the medium and is retained by the cells.

Although we do not know if the active material associated with the cells is a protein or a ligand carried by a protein, separation of the *PiE* cellular proteins in an isoelectric focusing gradient (unpublished observations) revealed a fraction that promotes yolk formation and egg production and suggests that the material is at least associated with proteins (Shapiro and Ferkovich, 2002). If it is a protein, it appears unlikely that its only benefit in the diet is to provide amino acids for structural proteins in oocyte development because it is active in the diet at such low concentrations. A significant effect on oviposition relative to the *Artificial Diet* was first observed at the 0.25 ml dose (31 µg protein) and continued to increase up to the 1.0 ml dose (124 µg protein). At the 1.0 ml dose, the increase in egg production started to decline. This decline in egg-laying activity could have been due to a toxicant present at the higher concentration, or to aggregation (insolubility) of the protein at the higher concentration, causing it to precipitate out of the diet. Another possibility is that it could have been due to some other factor or nutrient in the diet required in egg production that became limiting.

The effect of the cell protein on oviposition suggests that the *PiE* cell line, which was originally derived from embryos of *P. interpunctella*, has retained a differentiated function in culture by producing products similar to those produced in the *Plodia* egg. At the highest

concentration of protein tested, the PiE cells also improved survival of the females. Thus, the cells may produce a number of unknown nutrients or factors required by *O. insidiosus* for optimum growth and reproduction. Growth factors produced by insect cell lines have been documented in several studies (Ferkovich and Oberlander, 1991; Ferkovich et al., 1987; Hu et al., 1999; Loeb and Jaffe, 2002; Loeb et al., 1999), but this is the first time a cell line-supplemented diet has been shown to positively affect egg laying of an entomophagous insect. Hu et al. (1999) stated that cell-conditioned medium could exert a positive effect by providing growth factors, altering amino acid content, shifting pH, or releasing degradative enzymes.

Suboptimal fecundity in entomophagous insects reared on artificial diets is a common problem and barrier in implementing economical large-scale production of predatory insects for augmentative biological control (Grenier et al., 1994). Our studies demonstrate that insect cells have the potential of being used as a dietary supplement for artificial diets of *O. insidiosus* and possibly other species of *Orius* reared on artificial diet. An artificial diet could substitute for costly insects eggs used to produce *Orius* species. *O. insidiosus* is generally reared on eggs of *E. kuehiella* which are expensive, costing approximately \$1400/kg of fresh, irradiated eggs (Koppert Biological Systems; personal communication). Because the cells can be freeze-dried and still retain their fecundity-promoting activity (unpublished observation) they could be shipped and stored easily until needed. Although the cost of growing the cells is currently expensive because of the fetal bovine serum added to the medium, adapting the cells to chemically defined media could render the medium more affordable as a supplement for artificial diet. Finally, we anticipate that the *Plodia* PiE cell line and the abbreviated bioassay we used in this study will prove beneficial in purification and characterization of the oviposition-promoting material for *O. insidiosus* because the fractions can be assayed for oviposition-promoting activity in 1 week, compared with maintaining the insects on the diets for longer periods.

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